Manual on vegetative and micropropagation of mangroves

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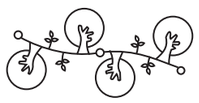

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We are thankful to the Forest Departments of Tamil Nadu and Orissa for providing necessary permission for the field work and sharing plant materials for experiments.

ABBREVIATIONS

$(NH_4)_2SO_4$	Ammonium Sulphate
10%	10 g of Sodium carbonate (Na_2CO_3) dissolved in 80 ml distilled water and made up to 100ml
1N HCl	8.77ml of 35%HCl (Generally Available) made up to 100ml using double distilled water.
1N NaOH	4g of Sodium hydroxide in 80 ml of double distilled water and made up to 100ml
2ip	6-(γ, γ - Dimethyl allyl-Amino) purine Riboside
Agar and Phytagel	Used for solidification of tissue culture media
BA	Benzyl Adenine
$Ca(NO_3)_2.4H_2O$	Calcium Nitrate Tetrahydrate
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
$CoCl_2.6H_2O$	Cobalt Chloride Hexahydrate
$CuSO_4.5H_2O$	Copper Sulphate Pentahydrate
Explant	Any plant part used in tissue culture
$FeSO_4.7H_2O$	Ferrous Sulphate Heptahydrate
H_3BO_3	Boric Acid
$HgCl_2$	Mercuric Chloride
Hormone	Growth regulating substance in plants
Humidifier	Mist forming instrument in mist chambers
IAA	Indole Acidic Acid
IBA	Indole Butyric Acid
K_2SO_4	Potassium Sulphate
KH_2PO_4	Potassium Dihydrogen Phosphate
KI	Potassium Iodide
KNO_3	Potassium Nitrate
$MgSO_4.7H_2O$	Magnesium Sulphate Heptahydrate
$MnSO_4.4H_2O$	Manganous Sulphate Tetrahydrate
Na_2CO_3	Sodium Carbonate
$Na_2EDTA.2H_2O$	Ethylene Diamine Tetra Aceticacid Disodium Salt
$Na_2MoO_4.2H_2O$	Sodium Molybdate dihydrate
$Na_2WO_4.2H_2O$	Sodium Tungstate
NAA	Naphthalene Acetic Acid
NH_4NO_3	Ammonium Nitrate
pH (-log[H+])	Denotes the concentration of hydrogen ions in a solution
ppm	parts per million
Sphagnum moss	A plant body creeping on the surface of a rock/soil in hilly areas
Tween-20	Surfactant used for surface sterilization of explants
v/v	Volume by volume
w/v	Weight by volume
$ZnSO_4.7H_2O$	Zinc Sulphate Heptahydrate

FOREWORD

The multiple benefits conferred by Mangrove wetlands in coastal estuarine areas are now widely recognized. For the families living along the coast, mangrove forests provide medicine, firewood, sustainable fish yield and protection against coastal storms. In the future, mangroves will assume greater ecological importance in view of a potential rise in sea level as a result of global warming, since they can tolerate varying degrees of coastal salinity. In addition, in the emerging era of genomics, proteomics and genetic modification, mangrove species constitute a source of valuable genes for a variety of useful traits including salinity tolerance.

Unfortunately, many precious mangrove wetlands have either already been destroyed for alternate uses including tourism and aquaculture or are under varying degrees of anthropogenic pressures. Where cyclones are frequent, coastal communities are becoming aware the value of mangrove forests, as for example in coastal Orissa. There is therefore a growing interest in coastal communities in the rehabilitation of degraded mangrove ecosystems. What is often lacking is the availability of planting material of the desired species. The present publication is designed to provide operational procedures for undertaking the multiplication and planting of mangrove trees in coastal estuarine areas. Both vegetative propagation and micropropagation methods are described.

In social forestry programmes, local communities are enabled to raise nurseries of forest tree species on the basis of a buy-back arrangement with Forest Departments. A similar programme is needed in the case of mangrove tree species. Local communities including school children can be assisted to raise mangrove nurseries. The planting material can be used in land belonging to forest and revenue departments as well as to local communities. This will help to speed up the restoration of degraded mangrove wastelands.

I congratulate Mr. P. Eganathan and Dr. C. Srinivasa Rao in bringing out a practical manual on mangrove propagation. I also thank Prof. P. C. Kesavan, DAE-Homi Bhabha Chair and Dr. V. Selvam for providing overall guidance and support for this work. I hope this manual will help to launch a community mangrove afforestation programme.

M. S. Swaminathan

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MANUAL ON VEGETATIVE AND MICROPROPAGATION OF MANGROVES

PART A

VEGETATIVE PROPAGATION

1.0 INTRODUCTION

1.1 Definition

Vegetative propagation is a method of producing plants identical in genotype with the mother plant. It is a method of producing large number of plants from the vegetative part of a mother plant. Any part of the plant such as stem, leaf, propagule and root can be used to produce plants through vegetative propagation. It is an asexual method of propagation. It is different from producing seedlings from the seeds, which is through sexual method of propagation.

The vegetative propagation forms an integral part of tree improvement programme. In this approach, the best planting stock with highest genetic quality can be obtained, which is not always possible with the sexually propagated progenies. Another advantage is that, by this technique, plants can be raised almost throughout the year and the plantable stock for some species can be obtained in shorter time than those raised through seeds.

1.2. Need for vegetative propagation:

- Vast areas of coastal wetlands potential for mangrove growth could be covered with mangrove vegetation for domestic and commercial utility. To achieve this, large scale production of planting material could be produced through vegetative propagation
- In many mangrove ecosystems planting season does not coincide with the reproductive season of the mangrove plants and in such situations, vegetative propagation would be ideal to supply planting material round the year
- Endangered species can be easily multiplied through vegetative propagation
- Propagation of sterile hybrids is possible mainly through vegetative propagation

1.3. Advantages and limitations of vegetative propagation

Advantages :

- Single stock can provide large number of plants
- The clones offer the advantages of genetic uniformity

- Seedlings produced through vegetative propagation take lesser time to develop, therefore, it is normally quicker and cheaper
- Multiplication of desired hybrids is easier without loss of desirable genes
- Helps to utilize maximum genetic gains of potential species in a shortest time
- Commercialization of planting material is made attractive

Disadvantages:

- Only a few species are amenable for vegetative propagation
- Standardization of methodology is time consuming and sometimes expensive
- Vegetative propagation is easier with young rather than old trees

1.4. Types of Vegetative Propagation

1. Stem cutting
2. Propagule cutting
3. Air layering

1.4.1. Stem cutting

Production of saplings from stems and/or branches of plants is called vegetative propagation by stem cuttings. Stem and/or branches of plants cut into small pieces ranging from 12 to 20cm in length with 3 to 5 or several nodes are known as "stem cuttings". Stem cuttings are divided into three categories viz., i). Soft wood (tender branches) ii). Semi-soft wood (intermediate of soft wood and hard wood) and iii). Hard wood (tertiary or secondary branches).

1.4.2. Propagule cutting

A propagule (hypocotyl) is a seed germinated in the mother tree itself. It is an unique feature of mangrove plants. A propagule contains different parts such as pedicel, fruit, collar and radicle. In a ripened propagule, plumules can be seen when the fruit is removed. Viviparous propagules are produced in all the Rhizophoraceae species of mangroves. The mature viviparous propagule (black or brown or mixture of both) can be collected from the mother tree and cut into small pieces of 2 to 7 cm. This is called "propagule cutting", which could be used for vegetative propagation (Plate 1, i to vi).

1.4.3. Air layering

Air layering is another method of vegetative propagation popularly called as "Chinese Layering". In this method, roots are produced in small branches by applying root producing hormones and rooting media. This method can be followed in tertiary branches without much damage to the mother plant.

2.0. MANGROVE PLANTS

2.1. Species composition

The mangrove species are a group of plants possessing specialized adaptive features to live in a saline, marshy estuarine environment where both physical and chemical properties vary widely both in space and time. There are about 30 families, 49 genera and 80 species available in India. Table 1 gives the list of common mangrove species in India.

Table 1 : Common Mangrove Species of India

Species	Family
Acanthus ilicifolius Linn.	Acanthaceae
Aegiceras corniculatum (Linn.) Blanco	Myrsinaceae
Amoora cucullata Roxb.	Meliaceae
Avicennia marina (Forsk.) Vieh.	Avicenniaceae
Avicennia officinalis Linn	Avicenniaceae
Bruguiera cylindrica (Linn.) Bl.	Rhizophoraceae
Bruguiera gymnorrhiza (Linn.) Lam.	Rhizophoraceae
Bruguiera parviflora (Roxb.) W.& R. ex Griff	Rhizophoraceae
Cerbera manghas Linn	Apocynaceae
Cerbera odollam Gaertner	Apocynaceae
Ceriops decandra (Griff.) Ding Hou	Rhizophoraceae
Dalbergia spinosa Roxb	Fabaceae
Derris trifoliata Lour.	Fabaceae
Excoecaria agallocha Linn.	Euphorbiaceae
Heritiera fomes Buch.-Ham.	Sterculiaceae
Heritiera littoralis Dryand.	Sterculiaceae
Intsia bijuga (Colebr.) O.Kuntze	Caesalpiniaceae
Kandelia candel (Linn.) Druce.	Rhizophoraceae
Lumnitzera racemosa Willd.	Combretaceae
Rhizophora apiculata Bl	Rhizophoraceae
Rhizophora hybrid	Rhizophoraceae
Rhizophora mucronata Poir.	Rhizophoraceae
Rhizophora stylosa Griff.	Rhizophoraceae
Sonneratia apetala Buch.-Ham.	Sonneratiaceae
Xylocarpus granatum Koen.	Meliaceae
Xylocarpus mekongensis (Prain) Pierre.	Meliaceae
Xylocarpus moluccensis (Lamk.) Roem.	Meliaceae

All the above species are capable of tolerating wide range of salinity, although the extent of tolerance varies widely. On the basis of the salt-tolerant mechanism,

mangrove plants are divided into salt excretory, in which excess salt is excreted through salt glands (e.g. *Avicennia* spp.), salt excluders, in which excess salt is excluded in the root zone itself (e.g. *Rhizophora* spp) and salt accumulators in which salt is accumulated in fleshy leaves (e.g. *Suaeda* spp.). In most of the species flowering is round the year but propagules and seeds are available only from September to January when the salinity is low due to heavy fresh water inflow. The utility of common mangrove species is presented in Table 2.

Table 2 : Important mangrove species in India and their utility

Species	Family	Time of flowering	Economic value
Avicennia marina	Avicenniaceae	Jun-Sep	Fodder, green manure, glue and firewood
Avicennia officinalis	Avicenniaceae	Jun-Sep	Fodder, green manure, glue and firewood
Bruguiera cylindrica	Rhizophoraceae	Round the year	Firewood and fodder
Bruguiera gymnorrhiza	Rhizophoraceae	Round the year	Firewood and fodder
Ceriops decandra	Rhizophoraceae	Round the year	Firewood and fodder
Cerberas manghas	Apocynaceae	June-Sep	Firewood and medicinal
Excoecaria agallocha	Euphorbiaceae	Feb-Sep	Plant parts used for the treatment of ulcers, leprosy, rheumatism, paralysis
Heritiera fomes	Sterculiaceae	Apr-Aug	Timber, scaffolds, boat building and paper industry
Heritiera littoralis	Sterculiaceae	Jan – Aug	Fiber, timber and firewood
Intsia bijuga	Caesalpaniaceae	June-Sep	Valuable timber
Kandelia candel	Rhizophoraceae	Round the year	Firewood, fodder and tannin
Rhizophora apiculata	Rhizophoraceae	Round the year	Firewood, tannins, leaves used for treating asthma.
Rhizophora hybrid	Rhizophoraceae	Round the year	Timber, firewood. tannins and adhesive, Decoction of the bark is used for diarrhoea, dysentery and leprosy
Rhizophora stylosa	Rhizophoraceae	Round the year	Firewood, tannins
Sonneratia alba	Sonnertiaceae	Feb-Oct	Firewood, paper pulp, matchwood; fermented juice is used to check hemorrhage
Xylocarpus granatum	Meliaceae	Jun – Sep	Timber, tannins, seed oil used as to lit lambs and firewood
Xylocarpus mekongensis	Meliaceae	Jul – Oct	Timber, tannins, oils extracted from seeds used for nerve disorder and antihelminthetic
Xylocarpus moluccensis	Meliaceae	Jul – Oct	Timber, tannins and firewood

3.0 PLUS TREE

3.1 Definition

A Plus tree is an individual tree of a species possessing superior morphological and reproductive characters than other individuals of the same species.

3.2. Identification of plus trees:

The following characters could be used to identify plus trees. Selection of plus tree should be site specific since plant characters, both quantitative and qualitative will vary from place to place.

a. Morphological characters
- Plant height
- Girth of the main stem at breast level
- Number of leaves per gram weight
- Canopy structure and Canopy area (Open, Closed)
- Number of pneumatophores per square meter
- Height of pneumatophores
- Number of stilt roots per square meter
- Length of the stilt roots
- Diameter of the stilt roots (surface area)
- Number of lenticels per square centimeter

b. Characters based on physiognomy
- Pests and disease damage
- Tidal inundation (frequency and duration)
- Salinity levels (soil and pore water salinity)
- Associate species

c. Reproductive characters
- Number of buds per inflorescence
- Number of flowers per inflorescence
- Number of fruits per inflorescence
- Number of propagules per inflorescence
- Number of propagules per tree
- Net weight of individual propagule
- Germination capacity of the propagules
- Germinability of viviparous propagules
- Viability of seeds
- Length of the propagules

d. Adaptation characters
- Photosynthetic efficiency
- Biomass production
- Soil salinity
- Adaptation to different types of soils
- Pollution tolerance
- Grazing effects

e. Commercial value
- Forest products, used by local people
- Timber
- Building material
- Fodder
- Fuel wood

3.3. Plus trees of mangrove species in Pichavaram

An example of quantitative and qualitative characters of the plus tree of different species of mangroves identified in the Pichavaram mangrove wetlands of Tamil Nadu is given Table 3.

Table 3 : Morphological and phenological characters of plus trees of *Avicennia* and *Rhizophora* identified at Pichavaram

Characters	*A. marina*	*A. officinalis*	*R. apiculata*	*R. mucronata*	*R. hybrid*
Morphological characters					
Plant height (m)	5.64	7.2	5.42	5.7	15
Girth of the main stem at breast level (cm)	75.7	134.6	25	27.5	46
Number of leaves per gram weight	0.579	1.273	2.666	7.407	4.878
Canopy area (m)	4.5	7.6	5	5.2	14
Canopy structure (Open, Closed)	Closed	Open	Closed	Closed	Closed
Number of pneumatophores / m^2	301	179	—	—	—
Height of pneumatophores (cm)	27.6	22.4	—	—	—
Number of stilt roots/ m^2	—	—	88	94	182
Length of the stilt roots (m)	—	—	3.61	3.6	4.3
Surface area of pneumatophores and diameter of the stilt roots (cm)	2.3	1.8	4.5	4.4	6.3
Number of lenticels/ cm^2	9	10	4	4	4
Reproductive characters					
Number of buds per inflorescence	21	34	6	8	9
Number of flowers per inflorescence	6	19	2	2	3
Number of fruits per inflorescence	16	22	—	—	—
Number of propagules per inflorescence	—	—	2	3	—
Number of propagules per tree	—	—	50	256	rarely 1to7 from total trees
Net weight of individual propagule (g)	8.169	10.822	40.7 to 48	97 to 151	79 to 98.5
Germination of propagules (%)	69.4	58.7	98	100	100
Length of the propagules cm)	—	—	14 to 20	40 to 56	23 to 37
Tolerance to grazing effects	High	High	—	—	—

4.0 VEGETATIVE PROPAGATION OF SPECIES IN RHIZOPHORACEAE

The species under the family to Rhizophoraceae are important in mangrove ecosystem. The genus *Rhizophora* encompasses evergreen trees having strong stilt roots and long propagules. The stilt roots of *Rhizophora* spread laterally and are buried deep in the mud, providing additional support to the tree. In view of this character, these trees are able to withstand high cyclones. Secondly, the root zones provide microhabitat for the juveniles of fish and prawn to grow. These species have become endangered in the mangrove of West Bengal. In the Pichavaram mangrove wetlands three *Rhizophora* species viz., *Rhizophora apiculata*, *R. mucronata* and *Rhizophora* hybrid are present. Generally, the maternal and paternal parents of the sterile hybrids are *R. mucronata* and *R. apiculata* respectively.

4.1. Propagule cutting

Materials:

Mature propagules, distilled water, solution to remove phenolics, growth hormones, refrigerator, knife, mist chamber, humidifier and mud filled polythene bags.

Method :

Step 1. Collection of propagules

Collect mature propagules from the plus trees identified based on the characters indicated in Chapter 3.0 Good propagules are fleshy, shining with red colored collar.

Step 2. Cutting of propagules

Cut the collected propagules into 2 to 5 cm pieces using a clean and sharp knife. The entire propagule can be used for cutting (Plate 1 A)

Step 3. Removal of phenolic compounds

Phenolics are a group of compounds present mostly in the bark and wood of almost all the mangrove species. Phenolics are essential compounds for the survival of mangrove plants in extreme environmental conditions, since they regulate growth and other physiological functions. However, in vegetative propagation they act as inhibitors in the formation of roots and shoots and hence, phenolics from propagule cuttings are to be removed before further processing.

The following method describes the preparation of solutions and treatment methods to remove the phenolic compounds. Removal of phenolic compounds involves short-term and long-term treatments.

- **Preparation of stock solution :** Take 20 g of Sodium carbonate (Na_2CO_3) and 20g of Sodium tungstate ($Na_2WO_4.2H_2O$), dissolve successively in 80 ml distilled water and make up the final volume to 100ml. It is a 20% stock solution.

- **Preparation of working solution (10% and 5%):** Take 50ml from the 20% stock solution and add 50ml of distilled water. This gives a 10% working solution. Take another 25ml of stock solution and add 75ml of distilled water. This gives a 25% working solution.

- **Short-term treatment to remove phenolic compounds :** Take 10% solution in small cups. Keep the basal portion of the cuttings immersed in the solution for 5-10 minutes. Wash the treated cuttings in distilled water two to three times (Plate 1 B).

- **Long-term treatment to remove phenolic compounds :** Keep the cuttings treated in 10% solution, for about 20-30 minutes in 5% working solution for final treatment. Wash the treated cuttings in distilled water two to three times. Now the propagule cutting is ready for hormone treatment.

Step 4. Hormone treatment

Hormone treatment is necessary to induce root formation. Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) are commonly used as root promoters. These promoters are available in many of the agro shops in the form of powder.

- **Preparation of hormone stock solution :** Take 1g of IBA (*Himedia*, Mumbai) and add little drops of 1N NaOH until it dissolves, and make it up with distilled water to 100ml. The strength of this stock solution is 10,000 ppm.

- **Preparation of hormone treatment solution :** The following table shows the method of preparing treatment solutions of different concentrations from the stock solution

Table 4 : Preparation of hormone treatment solutions of different concentrations

Quantity of stock solution (ml)	Quantity of distilled water (ml)	Concentration (ppm)
10	90	1000
15	85	1500
20	80	2000
25	75	2500

- **Hormone treatment of the cuttings :** Propagule cuttings treated to remove phenolics are again dipped in root promoting IBA hormone for 10-30 minutes. Since the concentration of IBA required for root promotion differs from species to species, propagule cuttings should be dipped in solutions of different concentrations as shown in Table 5.

Table 5 : Optimum concentration of root promoting hormones for maximum root initiation

Species	Growth Hormone	Concentration (ppm)	Rooting (%)
Bruguiera cylindrica	IBA	500	83
Bruguiera gymnorrhiza	IBA	700	75
Bruguiera parviflora	IBA	500	90
Bruguiera sexangula	IBA	500	93
Ceriops decandra	IBA+NAA	500+200	79
Kandelia candel	IBA+NAA	1000+500	95
Rhizophora apiculata	IBA	1500	93
Rhizophora mucronata	IBA	2000	89
Rhizophora x hybrid	IBA	1500	75

Step 5. Planting propagules in poly-bags/plastic pots :

After hormone treatment, cuttings are planted in poly-bags containing sand and clay at the ratio of 2:8. Keep planted cuttings in the mist chamber at 28±2°C and 70-80% relative humidity.

Step 6. Hardening the cuttings in mist chamber :

Cuttings are continuously monitored and maintained in controlled environment in the mist chamber. Roots will form after 20 to 25 days and shoots will form after 30 to 35 days (Plate 2). Water is sprayed 3 to 5 times/day inside the chambers to maintain the humidity and temperature.

Step 7. Hardening the saplings in nursery :

After 2 months, successfully established saplings should be kept under shady areas in nursery for 3-4 months. Before 25 days of field transfer treat the saplings

with saline water with maximum salinity level of 20 ppt of diluted sea water, or mangrove water itself or water in which common salt is dissolved for desired concentration (Plate 3).

Step 8. Planting in the field:

After successful hardening, saplings are ready for planting. At the end of the monsoon, transfer and plant the saplings in the prepared field.

4.2. Air-layering

Materials :

Growth hormones, distilled water, knife, syringe with needle, brush, sphagnum moss, rooting medium (clay: sand: soil), threads, and polythene.

Methods :

Step 1. Selection of branches:

Select semi-hard wood and hard wood branches of plus trees. Avoid selecting branches that are drooping too much and twigs that are very young or tender.

Step 2. Removal of bark of selected branches:

Remove the outer bark of the selected branch at 2 to 5 cm below the node. The portion of the branch where the bark is removed is called, wounded portion. Make a bridge of bark of 2 to 4mm thickness to connect the upper end of the mother plant and the lower end of the daughter plant (offspring) to be produced. This bridge is necessary for the maintenance of some of the important physiological functions, since in mangrove species formation of roots is very slow.

Step 3. Preparation of root promoting hormone:

Method is explained in under 4.1 Step 4 .

Step 4. Applying root-promoting hormones:

Apply the hormone all around the wounded portion using a fine brush. Hormone should be applied twice. Different concentrations of hormone are used for different species as shown in Table 6.

Table 6 : Optimum concentration of root-promoting hormone used in air-layering in different mangrove species for maximum rooting

Species	Hormone	Concentration (ppm)	Rooting (%)
Amoora cucullata	IBA+NAA	500+200	59
Avicennia marina	IBA	2500	42
Avicennia officinalis	IBA	2000	54
Cerbera manghas	IBA+NAA	1000+200	61
Cerbera odollam	IBA	1000	55
Excoecaria agallocha	IBA	2000	48
Heritiera fomes	IBA	2500	55
Heritiera littoralis	IBA	2000	51
Intsia bijuga	IBA	2000	46
Rhizophora apiculata	IBA	2000	48
Rhizophora mucronata	IBA	2500	52
Rhizophora x hybrid	IBA+NAA	1000+500	46
Sonneratia apetala	IBA	1500	45
Xylocarpus granatum	IBA	2000	56
Xylocarpus mekongensis	IBA	1500	64
Xylocarpus moluccensis	IBA	1000	60

Step 5. Applying rooting medium:

Prepare a mixture of sand and clay at 3:7 ratio and wet it with nearby mangrove water. This forms the first layer of the rooting medium. This first layer of rooting medium is followed by a layer of wet sphagnum mass (to retain moisture). Apply this medium of two layers around the wounded portion and finally cover it with a polythene cover and tie both the ends as shown in Plate 4. If shoot portion droops, the branch should be tied with nearby one to avoid drooping. Step 2 to step 5 are shown in Plate 4 A to F.

Step 6. Monitoring the air layering:

Periodically check the layered portion and whenever necessary, inject tap water through a syringe. Roots will be visible after 40-60 days.

Step 7. Hardening in growth chamber:

After the root system is well established, separate the rooted sapling from the mother plant using a sharp knife. Keep rooted saplings under mist chamber in the field nursery at 28°C and 70% relative humidity for 2 to 3 months. The procedure followed in hardening the saplings produced through propagule cuttings can be followed for hardening the saplings produced through air layering also.

Step 8. Planting in the field :

After 2 to 3 months, successfully established saplings should be treated with saline water of maximum salinity upto 20 ppt. Diluted sea water, or mangrove water itself or water in which common salt is dissolved for desired concentration could be used.

Results obtained in different species based on our studies are presented in Plates 5 and 6.

5.0. VEGETATIVE PROPAGATION OF OTHER SPECIES

This method described hereunder is applicable to the following species: *Acanthus ilicifolius, Amoora cucullata, Avicennia marina, Cerbera manghas, Excoecaria agallocha, Heritiera fomes, Heritiera littoralis, Intsia bijuga, Lumnitzera racemosa, Sonneratia apetala* and *Xylocarpus granatum.*

5.1. Stem cutting

Materials :

Tree twigs, solution to remove phenolics, growth hormones, refrigerator, knife, mist chamber, humidifier and mud filled polythene bags.

Methods :

Step 1. Collection of branches/stems :

Collect narrow/straight twigs from plus trees.

Step 2. Cutting of stems :

Select and cut stems of different types such as soft wood, semi-hard wood and hard wood; length of the cut stems may vary from 15 to 20 cm (Plate 7A).

Step 3. Removal of phenolic compounds :

The following method describes the preparation of solutions and treatment methods to remove the phenolic compounds. Removal of phenolic compounds involves short-term and long-term treatments.

- **Preparation of stock solution :** Take 20g of Sodium carbonate and 20g of Sodium tungstate, dissolve successively in 80 ml distilled water, and make up the final volume to 100ml. It is a 20% stock solution.

- **Preparation of working solution (10% and 5%) :** Take 50ml from the 20% stock solution, and add 50ml of distilled water. This gives a 10% working solution. Take another 25ml of stock solution and add 75ml of distilled water. It is a 5% working solution.

- **Short-term treatment to remove phenolic compounds :** Take 10% solution in small cups. Keep the basal portion of the cuttings immersed in the solution for 5-10 minutes. Wash the treated cuttings two to three times in distilled water.

- **Long-term treatment to remove phenolic compounds :** Keep the cuttings treated in 10% solution for about 20-30 minutes in 5% working solution for final treatment. Wash the treated cuttings two to three times in distilled water. Now the stem cutting is ready for hormone treatment

Step 4. Hormone treatment

Hormone treatment is necessary to induce root formation. Indole Acidic Acid (IAA), Indole Butyric Acid (IBA) and Naphthalene Acetic Acid (NAA) are commonly used as root promoters. These promoters are available in most of the agro shops in the form of powder.

- **Preparation of hormone stock solution :** Take 1g of IBA (*Himedia*, Mumbai) and add little drops of 1N NaOH until it dissolves, and make it up with distilled water to 100ml. The strength of this stock solution is 10,000 ppm.

- **Preparation of hormone treatment solution :** The following table (Table 7.) shows the method of preparing treatment solutions of different concentrations from the stock solution.

Table 7 : Preparation of hormone treatment solutions of different concentrations

Quantity of stock solution (ml)	Quantity of distilled water (ml)	Concentration (ppm)
10	90	1000
15	85	1500
20	80	2000
25	75	2500

- **Hormone treatment of the cuttings :** Stem cuttings treated to remove phenolics are again dipped in root promoting IBA hormone for 10-30 minutes. Since the concentration of IBA required for root promotion differs from species to species, stem cuttings should be dipped in solutions of different concentrations as shown in Plate 7 B. Table 8 shows the optimum concentration of the hormones for maximum rooting in the stem cuttings of different mangrove plants.

Table 8 : Optimum concentration of hormones for maximum rooting in the stem cuttings of different mangrove species

Species	Hormone	Concentration (ppm)	Rooting (%)
Acanthus illicifolius	IBA+NAA	500+1000	83
Amoora cucullata	IBA	1500	75
Avicennia marina	IBA	2000	56
Cerbera manghas	IBA	1500	63
Cerbera odollam	IBA	1000	69
Excoecaria agallocha	IBA	2000	68
Heritiera fomes	IBA	2500	72
Heritiera littoralis	IBA+NAA	1500+500	64
Intsia bijuga	IBA	2000	68
Xylocarpus granatum	IBA	2500	85

Step 5. Planting stem cuttings in poly-bags/plastic pots :

After the treatment, plant the cuttings in poly bags containing sand and clay. Keep planted cuttings under the mist chamber at 28±2°C and 70-80% relative humidity.

Step 6. Hardening the cuttings in mist chamber :

Monitor the cuttings continuously and maintain controlled environment. Roots can be seen after 20 to 25 days and shoots can be seen after 30 to35 days. Spray water inside the mist chamber 3 to 5 times/day, using hand sprayer, to maintain the humidity and temperature (Plate 7 C). Plates 8 and 9 show the rooting from stem cuttings in different mangrove species.

Step 7. Hardening the saplings in nursery :

After 2 months, keep the successfully established saplings under shady areas in the nursery for 3-4 months. Treat the established saplings with salt water up to 20 ppt for 25 days.

Step 8. Planting in the field:

After successful hardening, saplings are ready for planting. Immediately after the monsoon season, transfer and plant the saplings in the selected field.

PART B

MICROPROPAGATION

1.0 INTRODUCTION

1.1 Development of micropropagation protocols for mangrove plants

Micropropagation or plant tissue culture is a technology of growing isolated plant cells, tissues, organs or whole plants on semisolid or liquid synthetic nutrient media under aseptic and controlled environment. It is the most useful and widely used technology in tree improvement programmes. Mangroves are classical examples of plants, which have adapted to the shifting, saline and muddy environment. To fully adapt to this environment, mangroves have acquired a number of unique morphological, ecological and physiological characteristics. However, these special adaptive features make the mangrove plants recalcitrant to *in vitro* culture. There have been several inherent problems in the tissue culture of these species and hence, there has been only a few attempts as of now to propagate them through micropropagation. They are highly recalcitrant to the time tested media like Murashige and Skoog (1962) and Lloyd and Mc Kown (1981). The tissue browning occurs within few hours of inoculation, which limits the chance of survival of the explant tissue. There has been a high degree of contamination due to several microbial and fungal endophytes and the growth is very slow in the culture. The M.S.Swaminathan Research Foundation over came these problems and has established protocols for the first time for three species of mangroves viz., *Excoecaria agallocha, Avicennia officinalis* and *Acanthus ilicifolius*.

The micropropagation protocol developed for mangrove plants by the M.S.Swaminathan Research Foundation consists of a unique combination of macro nutrients while micro nutrient and vitamin composition are similar to regular MS media(Rao et al., 1998). The protocol is useful in propagating the plus tree of mangrove plants for ongoing mangrove afforestation programmes, keeping in view the alarming rate of mangrove forest degradation throughout the world.

1.2 Types of Micropropagation

There are two types of micropropagation techniques

a. Direct organogenesis
b. Indirect organogenesis

In direct organogenesis, stems with internodes are grown in culture media to produce multiple shoots and these shoots are removed and grown in rooting media. Once the rooting is established, the explants, which are now called as saplings, will be hardened in growth chamber, field nursery and then transferred to the field.

Production of saplings through indirect organogenesis involves the following steps. In the first step, a mass of undifferentiated cells is obtained from living tissues of plants in a culture medium. This mass of cells is called "callus". The callus is removed from the medium and grown in another medium to induce green shoots. The next step involves the removal and isolation of individual shoots, and growing them in a rooting medium. Finally the rooted saplings are hardened in growth chamber, nursery and then transferred to the field.

1.3 Advantages and limitations in micropropagation:

Advantages :

1. It is useful in rapid multiplication of plant material and can be used to produce both asexually (through plant parts) and sexually propagated (through seeds) plants.
2. Small pieces of plants (explants) can be used to produce a large number of plantlets in a small space.
3. Tissue culture provides a high degree of phenotypic uniformity.
4. Plantlets can be stored *in vitro* in a small space and less labour is required for maintenance of stock plants.
5. Plantlets produced through micropropgation are usually free from infection by bacteria, fungi and viruses.
6. Nutrient levels, light, temperature and other factors can be precisely controlled to accelerate vegetative multiplication and regeneration.
7. Tissue culture is independent of seasons. Tissue culture could be carried out round the year.
8. Plants *in vitro* require minimal attention between subcultures. Therefore, only limited labour and materials are required.

Disadvantages :

1. Chemicals used in medium preparation are expensive and less readily available.
2. High phenolics in mangrove plants delay the growth and thus the process becomes time consuming.
3. Growth in the culture medium is slow.
4. Endophytic fungal contamination is high in mangrove species.

2.0 MEDIA USED IN MICROPROPAGATION

The following are the culture media used in tissue culture: i) Murashige and Skoog (1968) medium or MS medium, ii) Woody Plan Medium or WPM medium (Lloyd and McKown, 1981) and iii) Schank and Hilderbrandt medium (1972) or SH medium. Apart from these, M.S.Swaminathan Research Foundation has developed a medium, for the tissue culture of mangrove plants, which is designated in the manual as X medium.

2.1 Composition of different culture media:

The following table (Table 9) shows the nutrient composition of the different culture media used in the micropropagation of mangrove plants. In all the three species of mangroves for which protocols have been developed, cultures are initiated in the X medium developed by the M.S.Swaminathan Research Foundation. MS medium can be used for the subculture of *Avicennia officinalis* and *Excoecaria agallocha* from second subculture onwards. SH medium can be used for shoot elongation in *Acanthus ilicifolius*. Half strength MS or WPM medium can be used for root initiation in *Avicennia officinalis* and *Excoecaria agallocha*.

Table 9 : Nutrient composition of different culture media

Nutrient composition	MS (1962)	WPM (1981) (mg/l)	SH (1972)	X (1998)
Major nutrients				
NH_4NO_3	1650	400	0	0
$(NH_4)_2SO_4$	0	0	300	500
KNO_3	1900	0	2500	525
$Ca(NO_3)_2.4H_2O$	0	556	0	0
$MgSO_4.7H_2O$	370	370	400	375
$CaCl_2.2H_2O$	440	96	200	200
KH_2PO_4	170	170	0	250
K_2SO_4	0	990	0	0
Iron stock				
$FeSO_4.7H_2O$	27.8	27.8	15	27.8
$Na_2EDTA.2H_2O$	37.3	37.3	20	37.3
Minor Nutrients				
$MnSO_4.4H_2O$	22.3	22.3	13.2	22.3
$ZnSO_4.7H_2O$	8.6	8.6	1	8.6
H_3BO_3	6.3	6.2	5.0	6.3
KI	0.83	0	1.0	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.1	0.1
$CuSO_4.5H_2O$	0.025	0.025	0.2	0.2
$CoCl_2.6H_2O$	0.025	0	0.1	0.1
Vitamins				
Inositol	100	100	100	100
Glycine	10	2	0	10
Thiamine.HCl	1	1	5	1
Nicotinic acid	1	0.5	5	1
Pyridoxine.HCl	1	0.5	5	1
Sucrose (g/l)	30	30	30	30
Agar (g/l)	8	8	8	8

2.2 Preparation of stock solution

In order to avoid delay in the preparation of the culture media, stock solutions of major and minor nutrients, iron and vitamins are prepared separately. These stock solutions can be stored at 4°C for about 6 months. Needed quantity of culture media are prepared whenever necessary by mixing these stock solutions and diluting them with double distilled water to get original concentration.

2.3 Preparation of 20X major nutrient solution:

To prepare 20X stock solution of major nutrients, dissolve all the chemicals except $CaCl_2.2H_2O$ one by one in about 500 ml of double distilled water as shown in Table 10. Stir the solution using a magnetic stirrer and ensure that all the chemicals are dissolved completely. Finally, add $CaCl_2.2H_2O$ and stir the solution till it is completely dissolved and make up the volume to 1000ml.

Table 10 : Composition of Stock solution of 20X major nutrients

Major nutrients	MS (1962)	WPM (1981) (g/l)	SH (1972)	X (1998)
NH_4NO_3	33	8	0	0
$(NH_4)\ _2SO_4$	0	0	6	10
KNO_3	38	0	50	10.5
$Ca\ (NO_3)\ _2.4H_2O$	0	11.12	0	0
$MgSO_4.7H_2O$	7.4	7.4	8	0
$CaCl_2.2H_2O$8.8	1.920	4	4	
KH_2PO_4	3.4	3.4	0	5
K_2SO_4	0	19.8	0	0

2.4 Preparation of 200X minor nutrient solution:

To prepare 200X stock solution of minor nutrients, dissolve all the chemicals one by one in about 500 ml of double distilled water as shown in Table 11. Stir the solution using a magnetic stirrer and ensure that all the chemicals are dissolved completely and make up the final volume to 1000ml.

Table 11 : Composition of stock solution of 200X minor nutrients

Major nutrients	MS (1962)	WPM (1981) (g/l)	SH (1972)	X (1998)
$MnSO_4.4H_2O$	4.46	4.46	2.64	4.46
$ZnSO_4.7H_2O$	1.72	1.72	0.200	1.72
H_3BO_3	1.26	1.24	1.0	1.26
KI	0.166	0	0.200	0.166
$Na_2MoO_4.2H_2O$	0.050	0.050	0.020	0.020
$CuSO_4.5H_2O$	0.005	0.005	0.040	0.040
$CoCl_2.6H_2O$	0.005	0	0.020	0.020

2.5 Preparation of 200X iron stock solution:

Table 12 gives the composition of the 200X iron stock solution. To prepare 500 ml of iron stock solution, first dissolve $FeSO_4.7H_2O$ in about 400 ml double distilled hot water. After ensuring that $FeSO_4.7H_2O$ is completely dissolved, add $Na_2EDTA.2H_2O$, stir the solution and make up the volume to 500ml. It is advisable to prepare only low quantity of iron stock solution since it will easily get contaminated. Discard the prepared solution if turbidity or precipitate is seen in the solution.

Table 12 : Composition of 200X iron stock solution

Iron Stock	MS (1962)	WPM (1981) (g/500 ml)	SH (1972)	X (1998)
$FeSO_4.7H_2O$ in grams	2.78	2.78	1.5	2.78
$Na_2EDTA.2H_2O$ in grams	3.73	3.73	2.0	3.73

2.6 Preparation of 1000X vitamin stock solution:

Table 13 gives the composition of 1000X vitamin stock solution. Weigh the vitamins accurately and dissolve them one by one in 80 ml of double distilled water. Once the vitamins are completely dissolved, make the final volume to 100 ml. Like iron stock, vitamin stock solution should also be prepared in less quantity, since it will also be easily contaminated. The stock solution should be used as early as possible.

Table 13 : Composition of vitamin stock solution

Vitamins	MS (1962)	WPM (1981) (mg/100ml)	SH (1972)	X (1998)
Glycine	1000	200	0	1000
Thiamine.HCl	100	100	500	100
Nicotinic acid	100	50	500	100
Pyridoxine.HCl	100	50	500	100

2.7 Other chemicals :

The following chemicals can be directly added to the culture medium:

i) Inositol 100mg/l
ii) Sucrose 30g/l
iii) Agar 8g/l or Phytagel 2g/l

2.8 Preparation of X culture medium :

To prepare 1 liter of X culture medium, take 600 ml of double distilled water and add 50 ml of the 20X stock solution of major nutrients, 5 ml of 200X stock

solution of micro nutrients, 5 ml of 200X iron stock solution, 1 ml of 1000X vitamin stock solution in succession and ensure that they are completely mixed. Add 100mg of Myo-inositol and 30 g of sucrose. Mix well using a magnetic stirrer until a clear solution is obtained. Add appropriate quantity of growth hormones such as IAA, IBA, NAA, Kn, BA and 2,4-D as shown in Table 14. Adjust pH to 5.75 to 5.85 by adding 1N NaOH or 1N HCl. Make the final volume to 1000 ml. Add 8 g of agar or 2 g of Phytagel. Cook the media on a hot plate until the agar or Phytagel is completely dissolved. Pour 3 to 5 ml of the cooked media into sterilized test tubes. Close the mouth of the test tube with sterilized cotton plug. Autoclave the test tubes for about 15 minutes at 121°C under 15 lb pressure. After autoclaving, keep the test tubes in a slanting position inside a sterile chamber.

2.9 Addition of growth hormones in culture media:

The experiments conducted in the M.S.Swaminathan Research Foundation showed that for successful shoot and root formation, different mangrove species require different growth hormones as shown in Table 14. These combinations and concentrations of growth hormones should be added to the culture and sub-culture media.

Table 14 : Combination and concentration of growth hormones to be used in culture and sub-culture media

Species	Growth hormone (ppm)				
	BA	Zeatin	IBA	2ip	IAA
E .agallocha					
Shoot induction (1st culture)	3	1	—	—	—
Shoot elongation (1st sub culture)	3	—	0.5	—	—
Rooting (2nd subculture)	—	—	0.5	—	—
Avicennia officinalis					
Shoot induction (1st culture)	1	—	0.5	—	—
Shoot elongation (1st sub culture)	—	—	—	—	—
Rooting (2nd subculture)	—	—	0.5	—	—
Acanthus ilicifolius					
Shoot induction (1st culture)	0.5	—	—	0.2	1.0
Rooting (1st sub culture)	—	—	0.5	—	—

3.0 Methods of micropropagation

Materials:

Stock solutions of MS, WPM, SH and X media, explants, glassware, inoculation chamber, sterilized culture racks, growth chamber

Methods :

Step1. Collect explants, preferably shoot portion, from the field or mist chamber or nursery. The explant material may be leaf segments, uninodal and binodal segments from mature trees as well as seedlings.

Step 2. Wash the explant in running tap water for 1 hour. This is necessary to remove the exudates (phenolics, tannins, and mucillages) present within the tissue.

Step 3. Again wash the explant with Tween 20 (2%, v/v) and rinse until traces of soap are removed. Take the explants to a sterile laminar flow and surface sterilize the explants with $HgCl_2$ (0.1%, w/v) followed by three washes with sterile distilled water.

Step 4. Trim the explants with sterilized knife and cut them into small pieces of leaf, uninodal and binodal segments. Cut the lower portion of the nodal explants at an angle of 20 to 30 degrees to get a slanting basal portion, which facilitates in effective absorption of nutrition from the medium.

Step 5. Transfer the trimmed explants to the culture media.

Step 6. Incubate the culture at 24± 2°C and 60% relative humidity under a 16-hour/day photoperiod. Provide light intensity of 50 µMol m^{-2} s^{-1} using a cool white fluorescent light.

Step 7. After shoot initiation, sub-culture the explants for shoot elongation and multiplication. For this purpose use different combinations and concentrations of growth hormones as shown in Table 14.

Step 8. After shoot elongation, remove the explants, cut the shoots in a sterile inoculation chamber, and transfer the shoots to rooting media for root initiation. Transfer the well established rooted plants into a growth chamber.

Step 9. Harden the rooted plants in growth chamber (e.g. NK System LP-1PH) at 80% relative humidity and 26°C for a period of three weeks.

Step 10. Transfer the hardened plants to the mangrove nursery and after hardening for about 2 to 5 months, treat them with different salinities ranging from 5 to 20 ppt.

Step 11. Transfer the hardened plants to the site selected for plantation.

3.1 Micropropagation of *Excoecaria agallocha*

- Binodal segments respond well in X medium with a combination of BA, Zeatin and IBA as shown in Table 14

- The X medium has an overall low mineral content with relatively high concentrations of SO_4^{2-}, NH_4^+, PO_4^- and K^+ ions. Auxiliary shoot induction is high in this medium compared to MS and WPM media
- Per cent shoot induction and mean shoot length is maximum (52%) when the X medium has BA, Zeatin and IBA. Addition of Zeatin in the culture medium (up to 1ppm) further increases shoot induction response (72%) with no significant effect on shoot length
- Binodal segments give a better shoot induction over uninodal segments. The shoot elongation rate enhances from the second subculture onwards (Plate 10A)

3.2 Micropropagation of *Avicennia officinalis*

- Uninodal explants of *A. officinalis* responded well in the X medium with a combination of BA and IBA
- There is an increase in the shoot induction response with the increase in BA and IBA concentrations to 1.0ppm and 0.5ppm respectively
- Rooting response is good when the regenerated shoots of 5 cm length are transferred to the X medium supplemented with 0.5ppm IBA
- After 2 weeks of rooting in the growth chamber, the plantlets can be transferred to the potting medium consisting of 1:1 garden soil and sand mixture. A high humidity condition is to be maintained for another 4 weeks (Plate 10B)

3.3 Micropropagation of *Acanthus ilicifolius*

- Uninodal explants of *Acanthus ilicifolius* cultured on SH medium supplemented with BA (0.5 ppm), 2ip (0.2ppm) and IAA (1ppm) show maximum shoot induction
- Shoot elongation can be achieved when the shoots are divided and subcultured on the SH basal medium supplemented with half of the above concentrations of hormones
- The individual elongated shoots subcultured on the ½ SH medium supplemented with 0.5ppm IBA produce healthy roots
- The rooted plants can be grown in pots with vermiculite in the growth chamber with 75% relative humidity and 26°C, to get maximum survival (Plate 11)

In the experiments conducted in the M.S.Swaminathan Research Foundation, 95% of the plantlets survived in the hardening chamber when the above procedures were followed.

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Plate 2 : Root and shoot development in propagule cuttings

A. *Bruguiera cylindrica*
B. *Ceriops decandra*
C. *Kandelia candel*
D. *Rhizophora mucronata*
E. *Rhizophora apiculata*
F. *Rhizophora* hybrid

Plate 7 : Stem cuttings in *Excoecaria agallocha*

A. Cutting of stem
B. Treating the stem cuttings with hormone
C. Development of stem cuttings into sapling

Plate 4 : Air-layering in *Excoecaria agallocha* in the field

A. Removal of bark
B. Application of root promoting hormone
C. Application of rooting media
D. Wrapping with polythene sheet
E. Closing the end of the wrapping
F. Air-layering

Plate 5 : Rooting through air-layering in different mangrove species

A. *Heritiera fomes*
B. *Excoecaria agallocha*
C. *Xylocarpus moluccensis*
D. *Rhizophora* hybrid
E. *Sonneratia apetala*

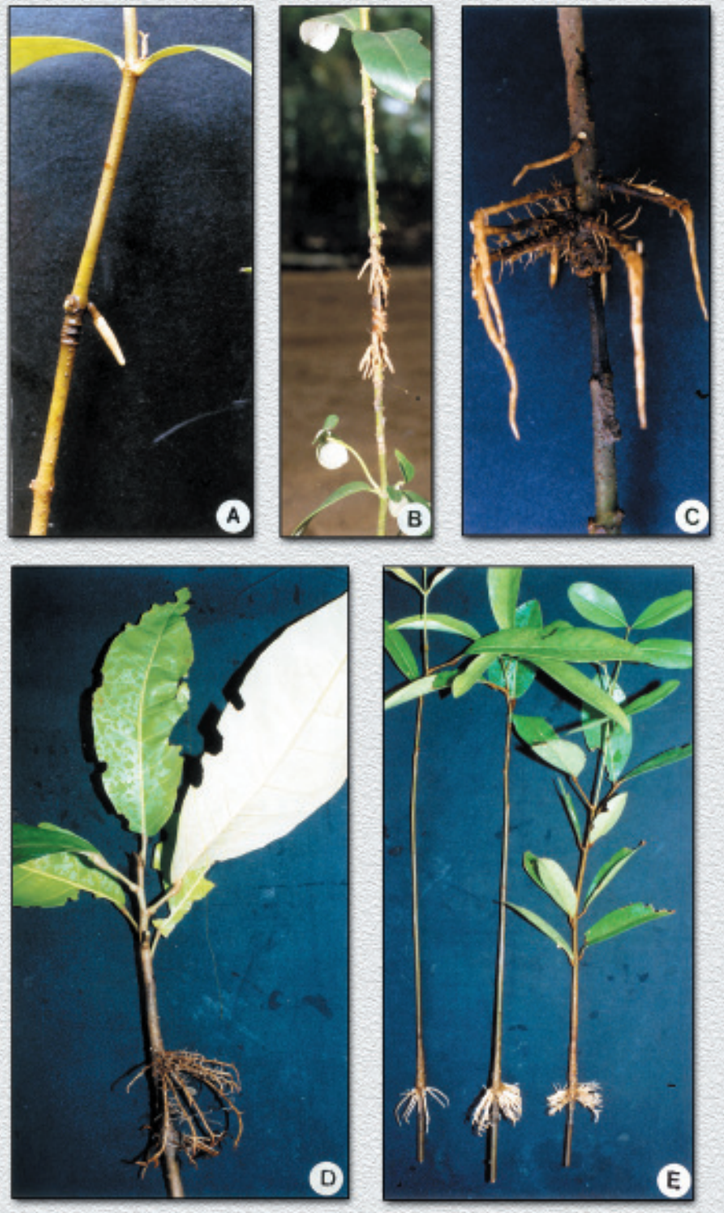

Plate 6 : Rooting through air-layering in different mangrove species

A. *Avicennia officinalis*
B. *Avicennia marina*
C. *Intsia bijuga*
D. *Heritiera littoralis*
E. *Xylocarpus granatum*

Plate 3 : Development and hardening of propagated plants in the nursery and mist chamber

A. General view of the field nursery with mist chamber
B. Stem cuttings of the *Acanthus ilicifolius*
C. Inside view of the mist chamber
D. Flow of mist inside the mist chamber

Plate 8 : Rooting in stem cuttings in different mangrove species

A. *Acanthus ilicifolius*
B. *Avicennia marina*
C. *Excoecaria agallocha*
D. *Heritiera littoralis*

Plate 1 : Propagules of different species of Rhizophoraceae

i. *Rhizophora mucronata*
ii. *Rhizophora apiculata*
iii. *Rhizophora* hybrid
iv. *Bruguiera cylindrica*
v. *Ceriops decandra*
vi. *Kandelia candel*

A. Propagule cutting - *Bruguiera cylindrica* **B. Hormone treatment**

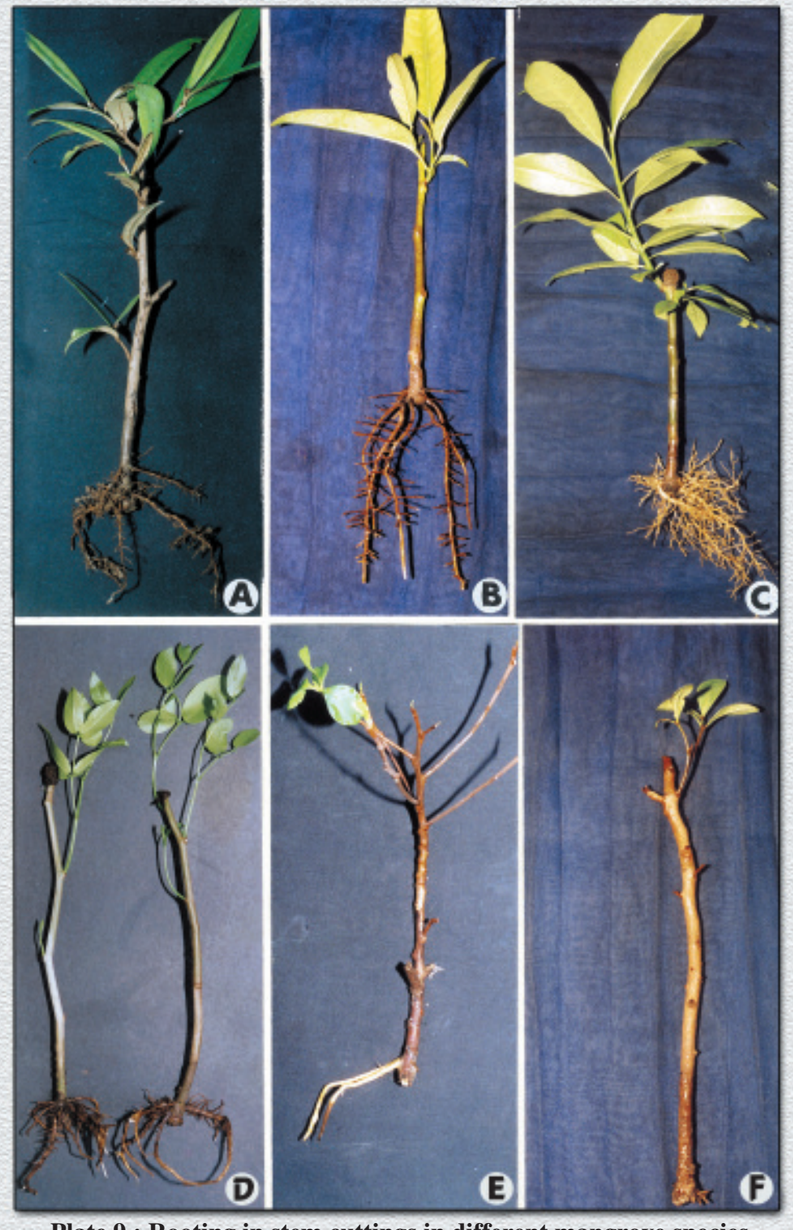

Plate 9 : Rooting in stem cuttings in different mangrove species

A. *Heritiera fomes*
B. *Amoora cucullata*
C. *Cerbera manghas*
D. *Intsia bijuga*
E. *Lumnitzera racemosa*
F. *Xylocarpus granatum*

Plate 10 : A. Micropropagation of *Excoecaria agallocha*

A. Uninodal explant B. Binodal explant
C. Rooting of shootings D. Hardening plants
E. Field transfer plant

B. Micropropagation of *Avicennia officinalis*

A. Uninodal explant shoot initiation B. Shoot elongation
C. Rooting of shoots D. Field transferred plant

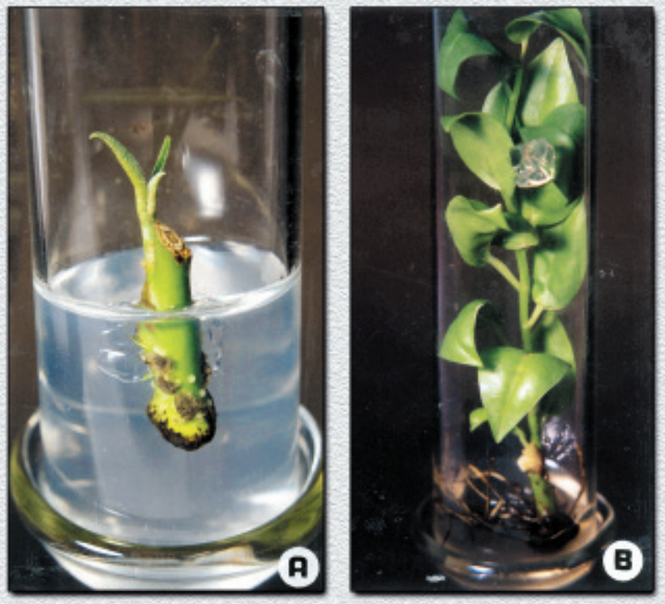

Plate 11 : Micropropagation of *Acanthus ilicifolius*

A. Uninodal with shoot initiation B. Rooting of shoots
C. Field transferred plant

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